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Effect of Exposure Route, Regimen, and Duration on Benzene-Induced Genotoxic and Cytotoxic Bone Marrow Damage in Mice

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Mice were exposed to benzene for 13 to 14 weeks by inhalation for either 3 or 5 consecutive days per week or by gavage for 5 consecutive days per week. A weekly evaluation of peripheral blood smears for micronucleated (MN) erythrocyte frequencies and for the percentage of polychromatic erythrocytes (PCE) indicated that the induction of MN-PCE by benzene depended on the sex and strain of mice and on the route of exposure, but not on the inhalation regimen or on the exposure duration. The frequency of MN normochromatic erythrocytes (NCE) not only depended on the sex and strain of mice and on the route of exposure, but directly depended on the inhalation regimen and on the exposure duration. Similarly, the extent of erythropoietic depression in benzene-exposed mice was dependent on sex, mouse strain, exposure duration, and route. However, in contrast to the MN-NCE data, the 3 day/week exposure regimen induced a more persistent depression in erythropoiesis than the 5 day/week exposure regimen. Exposure to benzene also induced in mice a significant depression in packed cell volume (PCV) and bone marrow cellularity, the magnitude of which depended on the sex and strain of mice and on the regimen and route of exposure.

Introduction

Benzene is used extensively in industry and commerce and presents many risks in regard to environmental contamination and human health (*1,2*). Exposure to benzene in man and animals results in a variety of adverse health effects, including an increased risk for cancer and aplastic anemia (*2-8*). In animal studies, benzene has been demonstrated to induce genotoxic and cytotoxic damage in bone marrow and, under acute exposure conditions, the magnitude of the damage depends on the dose, sex, strain, and species of the animal (*2,9,10*). However, few studies have evaluated the effect of exposure duration, regimen, or route on the levels of genotoxic damage induced by benzene under extended exposure conditions. Barale et al. (*11*) evaluated micronuclei (MN) frequencies in peripheral blood erythrocytes of CD-1 mice exposed to benzene over an 8-week period, whereas Choy et al. (*12*) evaluated MN frequencies in peripheral bood erythrocytes of B6C3F$_1$ mice treated with benzene for up to 2 years (*6*). In both studies, benzene was administered by gavage on 5 consecutive days per week, and MN analysis was limited to normochromatic erythrocytes (NCE). The conclusion in both studies was that the ability of benzene to induce bone marrow genotoxic damage declined with increasing exposure duration. However, since the frequency of micronucleated NCE depends on several factors, other interpretations of the data are possible.

To specifically evaluate the effect of exposure duration on the ability of benzene to induce genotoxic damage in murine bone marrow, MN frequencies in both NCE and polychromatic erythrocytes (PCE) were evaluated in peripheral blood (*13-15*). This approach, first introduced by MacGregor and his colleagues (*16,17*), permits in the same animal an evaluation of both recently induced and chronically accumulated bone marrow damage. Because of the lack of information on benzene-induced genotoxic damage under multiple exposure conditions, the study was also designed to evaluate sex and strain differences in response and to compare the magnitude of the damage induced by inhaled benzene with benzene given by oral gavage. In addition, because Irons (*18*) reported that intermittent treatments of mice with metabolites of benzene (hydroquinone and phenol) induced more bone marrow damage (i.e., loss in cellularity) than chronic treatments at the same doses, two exposure regimens were used in the inhalation study. An evaluation in the peripheral blood of the percentage of PCE among total erythrocytes was included to provide an assessment of the rate of erythropoiesis and thus a measure of bone

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marrow cytotoxicity (*19*). At the completion of the exposure period, two additional measures on bone marrow toxicity—a depression in packed cell volume and in bone marrow cellularity—were assessed.

Materials and Methods

Chemicals and Animals

Benzene (CAS No. 71-43-2), reagent grade, thiophene free, was obtained from Mallinckrodt Chemical Co. (Paris, KY). Acridine orange and reagent grade corn oil was obtained from Sigma Chemical Co. (St. Louis, MO).

Male DBA/2 and C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 9 weeks of age; male B6C3F$_1$ (C57BL/6 × C3H) mice were obtained from Frederick Cancer Research Center (Frederick, MD) as weanlings. After receipt, the mice were acclimated for a period of 2 weeks in standard laboratory stainless-steel cages with corn cob bedding (Bed O' Cobs, J. R. Nielson and Sons, Inc.). The population density was kept at no greater than one animal per 90 cm2, under controlled laboratory conditions [i.e., 22 ± 2°C, 50 ± 15% relative humidity, 20 air exchanges per hr and a 12-hr photoperiod (light: 0700–1900)]. Food (Purina Lab Rodent Chow 5001) and water were provided *ad libitum*. Coded toe clipping enabled identification of individual animals throughout the experiment.

Exposure Protocol

Groups of mice (n = 6–10) were a) exposed to either 300 ppm benzene or to ambient air (6 hr per day) for a period of 13 weeks, using one of two exposure regiments (regimen 1:5 consecutive exposure days per week; regimen 2:3 consecutive exposure days per week) (*14,15*); or b) given benzene orally (400 mg/kg diluted in corn oil, which was administered at a volume of 80 mL/kg) by gavage on 5 consecutive days per week for 14 weeks (*16*). Benzene at 300 ppm was used because of the ability of this concentration to induce adverse health effects (e.g., leukopenia, anemia, cancer) (*5,20–22*) and genotoxic damage (*23,24*) in exposed mice and because this concentration was not expected to induce overt mortality over the course of the study (*5*). Furthermore, 300 ppm benzene was believed to be within the linear portion of the dose range for the absorption of inhaled benzene in mice (*25*). Benzene at a dose of 400 mg/kg was used in the gavage study because this dose was estimated to exceed the total amount of benzene absorbed by a mouse during a 6-hr exposure to 300 ppm, because it induced close to the maximal MN-NCE response in B6C3F$_1$ mice in the National Toxicology Program (NTP) benzene cancer bioassay study (*12*), and because it was similar to the maximum dose used by Barale et al (*11*). The selection of the inhalation regimens was based on studies by Irons (*18*), in which greater cytotoxic damage occurred when mice were treated with various metabolites of benzene on 3 days per week as compared to 6 days per week.

DBA/2 and C57BL/6 mice were included in this study because these two strains are well known for their differential organ-specific carcinogenicity and toxicity following repeated exposures to polycyclic aromatic hydrocarbons (*26*) and because they differ in their sensitivity to benzene as judged by the induction of sister chromatid exchanges (SCE) (*23*), the inhibition of red blood cell formation (*27*), and the induction of micronucleated PCE in bone marrow (*28*). Also, following repeated but not acute exposures to benzene, DBA/2 mice exhibit a greater amount of water-soluble metabolites of benzene and covalent-binding in bone marrow than similarly exposed C57BL/6 mice (*27*).

B6C3F$_1$ were included to further evaluate and compare the sensitivity of the strain used in the NTP benzene cancer bioassay (*6*) against the other two strains. Female mice of one strain were included in the study because the genotoxic and carcinogenic activity of benzene has been shown to be highly sex dependent (*10*). The time-weighted average concentration of benzene for regimen 1 and regimen 2 in the inhalation study was 299.8 ppm (63 exposure days) and 300.4 ppm (39 exposure days), respectively. Additional details on the inhalation exposure study and on the gavage study can be found in Luke et al. (*13,14*) and Tice et al. (*15*), respectively.

Hematologic and Cellular Analyses

Peripheral blood smears were prepared weekly as described in Luke et al. (*13*) from all mice beginning with the week prior to the first week of exposure. Slides were fixed in absolute methanol and stained for analysis with acridine orange and scored as described in Luke et al. (*13*). In each animal at each sample time, 1000 PCE and 1000 NCE were scored for the frequency of micronucleated cells, and 1000 erythrocytes were evaluated for the percentage of PCE.

At the completion of the study, a single tibia was obtained from each mouse, cleaned of adhering tissue, and the marrow removed by repeatedly flushing the bone cavity with phosphate-buffered saline (PBS) (pH 7.4; ~10 mL). The marrow was pelleted, resuspended in PBS, and the total number of nucleated cells determined using a Coulter electronic counter (Model ZBI).

At the completion of the study, the packed cell volume (PCV) was measured using heparinized micro-hematocrit tubes (Clay-Adams, Inc., NY). The tubes were centrifuged (15,500 rpm) for 5 min in a hematocrit centrifuge (Drummond) and read in a hematocrit reader (Drummond).

Statistical Analysis

The alpha level was set at 0.05. Complete details of the statistical analyses of the MN and percent PCE data can be found in Luke et al. (*13,14*). Briefly, temporal averages for MN-PCE, MN-NCE, and the percentage of PCE were calculated for each animal by summing the values across time (except for the MN-NCE data, week 0 was omitted from the exposed mouse calculations) and divid-

ing by the number of samples evaluated. Using temporal averages, the data were evaluated for benzene, sex, strain, regimen, and route of exposure effects using a two-way Brown Forsythe analysis of variance (ANOVA) based on separate group variances (*29*). Group mean data were compared using Student's *t*-test based on separate variances after the alpha level had been Bonferroni corrected for the appropriate number of multiple comparison. Two-way ANOVA and Student's *t*-tests were also used to evaluate PCV and cellularity data. In cases where a signifcant strain and/or sex difference in control data was observed, the difference between control and exposed data were used in the statistical analysis. Student's *t*-tests were used to determine at which sample times the percentage of peripheral blood PCE in the benzene-exposed mice were significantly different from control values. To evaluate for an exposure duration-dependent alteration in the induction of genotoxic damage in the bone marrow of benzene-exposed mice, weekly frequencies of MN-PCE in each animal were analyzed by multiple regression analysis. As with the calculation of temporal averages, pre-exposure MN-PCE data were omitted from the analysis of benzene-exposed mouse data.

Results

Complete data at every sample time for MN-PCE, MN-NCE and the percentage of PCE in peripheral blood of each mouse in the inhalation study can be found in Luke et al. (*13,14*) and for the gavage study in Tice et al. (*15*)

MN-NCE Levels

There was an exposure duration-dependent increase in the frequency of MN-NCE in mice exposed to benzene by inhalation (Fig. 1) or given benzene by gavage (Fig. 2). In the inhalation study, mice exposed to benzene 5 days per week exhibited a significantly greater increase ($p < 0.0001$) in the frequency of MN-NCE than mice exposed 3 days per week (Table 1). Within each regimen of the inhalation study, the increase in MN-NCE depended on the sex (male > female; $p < 0.0001$) and on the strain (C57BL/6 ≅ B6C3F$_1$ > DBA/2; $p < 0.0001$) of mice. The frequency of MN-NCE in male B6C3F$_1$ mice administered benzene by gavage 5 days per week was significantly less ($p < 0.0001$) than that in male mice of the same strain exposed to benzene 5 days per week by inhalation. In male and female mice inhaling benzene 3 days perweek, the MN-NCE levels appeared to reach steady-state conditions by about 6 weeks of exposure (Fig. 1). In female DBA/2 mice inhaling benzene 5 days per week (Fig. 1) and in male B6C3F$_1$ given benzene by gavage (Fig. 2), similar kinetics in the rise and saturation of MN-NCE levels were observed. However, steady-state conditions were not attained over the duration of the study in male mice exposed to gaseous benzene on 5 days per-week. Strain differences in the kinetics of MC-NCE accumulation in these mice are readily apparent (Fig. 1). In male DBA/2 mice, the frequency of MN-NCE increased over the duration of the study while, in the other two strains of mice, MN-NCE frequencies peaked around week 6 of the inhalation exposure. This peak in frequency was followed by a decline over the following 4 to 5 weeks

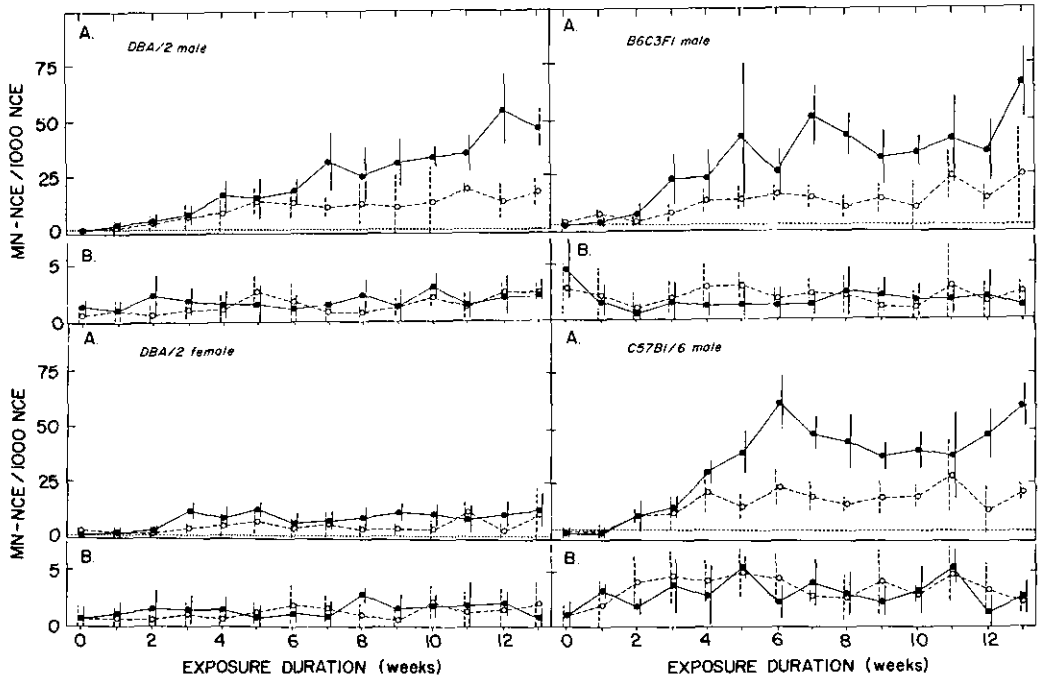

FIGURE 1. Frequency of peripheral blood MN-NCE across time in male DBA/2 mice, female DBA/2 mice, male B6C3F$_1$ mice, and male C57BL/6 mice exposed to 300 ppm benzene. (*A*) Data on benzene-exposed mice. The dashed line indicates the upper 95% confidence limit for MN-NCE frequency in control mice. (*B*) Data on control mice. (O—O) Mice exposed 3 days per week; (●—●) mice exposed 5 days per week. Error bars indicate 95% confidence limits.

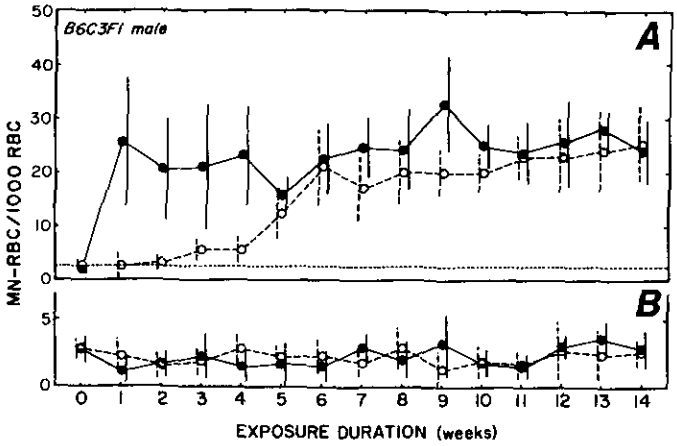

FIGURE 2. Frequency of peripheral blood MN-NCE and MN-PCE across time in male B6C3F$_1$ mice administered 400 mg/kg benzene by gavage. (*A*) Data on benzene-exposed mice. (*B*) Data on control mice. The dashed line indicates the upper 95% confidence limit for MN-NCE/MN-PCE frequency in control mice. (○—○) MN-NCE; (●—●) MN-PCE. Error bars indicate 95% confidence limits.

and then by a second increase in MN-NCE levels at the end of the study.

MN-PCE Levels

Exposure to benzene by inhalation or by gavage induced a highly significant increase ($p < 0.0001$) in the frequency of MN-PCE detected in the peripheral blood of mice (Figs. 2 and 3; Table 2). Within each strain used in the inhalation study, the increase in the frequency of MN-PCE was independent of regimen (p values between 0.07 and 0.54). After pooling MN-PCE data, where acceptable, between exposure regimens and correcting for strain differences in control frequencies, the ability of benzene to induce MN depended ($p < 0.0001$) on the sex (male > female DBA/2 mice) and the strain DBA/2 > C57BL/6 ≅ B6C3F$_1$) of mice and on the route of exposure (inhalation > oral). Except for B6C3F$_1$ mice exposed to 300 ppm benzene for 3 days per week, regression analyses of individual mouse MN-PCE frequencies against exposure duration revealed only an occasional animal with a significant time-dependent alteration. Among the male B6C3F$_1$ mice exposed to benzene 3 days per week, MN-PCE levels in three or the six mice had a significant positive slope when regressed against exposure duration. While not significant, the remaining three mice also had a positive exposure duration-dependent slope for MN—PCE response.

PCE Frequency

Exposure to benzene by either route or inhalation regimen initially induced a significant depression in the percentage of PCE in the peripheral blood of mice of all three strains (Figs. 4 and 5). The extent and duration of the depression depended on the sex, strain, and exposure regimen. Female DBA/2 mice exhibited the least initial suppression of erythropoiesis and the percentage of peripheral blood PCE returned to control levels by the third week of exposure. A two-way ANOVA of the female mice terporal averages prsented in Table 3 indicated that while benzene significantly depressed the percentage of PCE ($p = 0.0201$), the extent of depression was not dependent on exposure regimen ($p = 0.1523$). Although male mice of all three strains experienced a marked suppression of PCE production after the completion of the first or second week of inhalation exposure, the effect was more pronoucned in DBA/2 mice and more pronounced in mice exposed to benzene 3 days per week (Fig. 4). A two-way ANOVA of temporal averages (Table 3) revealed a significant difference between inhalation exposure regimens in C57BL/6 and male DBA/2 mice ($p < 0.05$) and, after correcting for strain differences in control data, a significant difference among strains ($p < 0.0001$) in the ability of benzene to suppress erythropoiesis in both regimens. Treatment with 400 mg/kg benzene by gavage initially resulted in a significant depression of

Table 1. Peripheral blood MN-NCE temporal averages in benzene-exposed mice.

Strain	Sex	Route[a]	Exposure regimen	Control[b] Mean SEM	*n*	Exposed[b] Mean SEM	*n*
B6C3F$_1$	M	Gav	5 day	2.20 ± 0.129	9	15.32 ± 2.341*	9
B6C3F$_1$	M	Inh	5 day	1.70 ± 0.186	6	30.73 ± 0.906*	6
B6C3F$_1$	M	Inh	3 day	1.97 ± 0.204	6	12.32 ± 0.522*	6
C57B1/6	M	Inh	5 day	2.93 ± 0.174	6	34.73 ± 1.290*	6
C57B1/6	M	Inh	3 day	3.28 ± 0.295	6	15.78 ± 0.593*	5
DBA/2	M	Inh	5 day	1.70 ± 0.157	6	23.47 ± 1.040*	6
DBA/2	M	Inh	3 day	1.35 ± 0.034	6	10.55 ± 0.633*	6
DBA/2	F	Inh	5 day	1.35 ± 0.102	6	7.72 ± 0.995*	6
DBA/2	F	Inh	3 day	1.15 ± 0.177	6	4.98 ± 0.497*	6

[a]Abbreviations: gav, gavage; inh, inhalation.
[b]Mean number of MN-NCE per 1000 NCE ± SEM among *n* mice.
*Significantly different from corresponding control values at $\alpha = 0.05$.

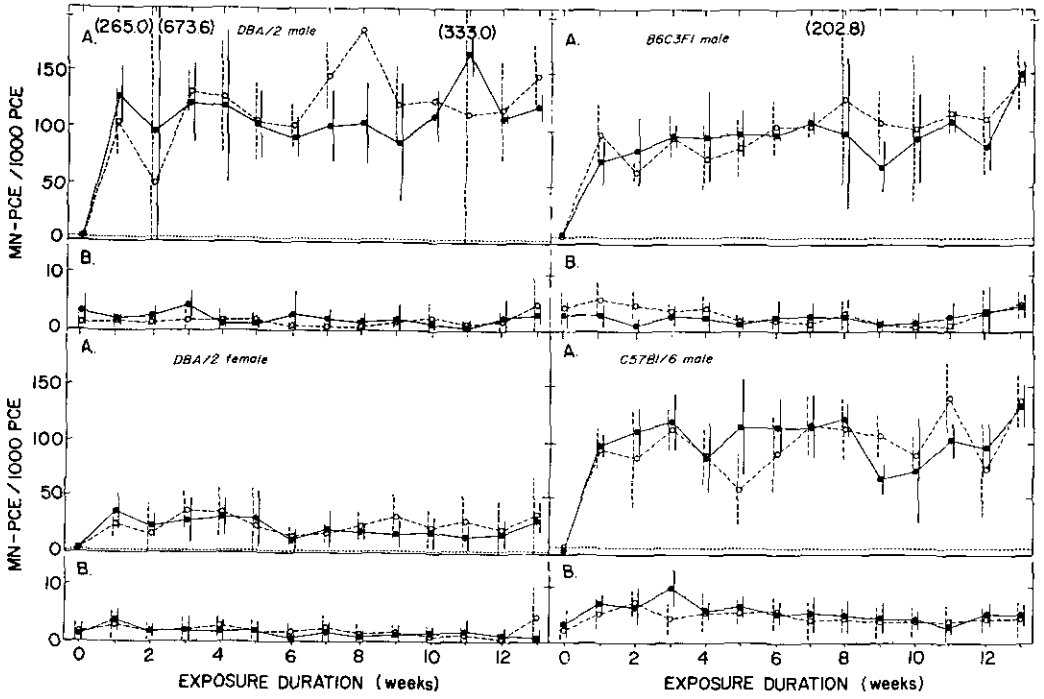

FIGURE 3. Frequency of peripheral blood MN-PCE across time in male DBA/2 mice, female DBA/2 mice, male B6C3F$_1$ mice, and male C57BL/6 mice exposed to 300 ppm benzene. (*A*) Data on benzene-exposed mice. The dashed line indicates the upper 95% confidence limit for MN-NCE frequency in control mice. (*B*) Data on control mice. (O—O) Mice exposed 3 days per week; (●—●) mice exposed 5 days per week. Error bars indicate 95% confidence limits.

PCE levels in the peripheral blood of male B6C3F$_1$ mice which persisted for only 2 weeks (Fig. 5). The lack of a significant depression in the percentage of PCE in gavage-treated mice, as determined by an analysis of temporal averages (Table 3), is due to the lack of persistence in the depression of erythropoiesis in these animals.

Packed Cell Volume

Exposure to benzene induced a significant depression ($p < 0.01$) in PCV in mice evaluated at the completion of the study (Table 4). In B6C3F$_1$ and C57BL/6 mice, the extent of depression was independent of the inhalation regimen. Exposure to benzene by inhalation induced a greater depression in male B6C3F$_1$ mice than benzene given by gavage. After correcting for differences in PCV levels in control animals, the extent of depression in mice exposed to gaseous benzene on 3 days per week depended on the strain (B6C3F$_1$ ≅ C57BL/6 > DBA/2; $p = 0.0163$), while in DBA/2 mice, the extent of depression was sex-dependent (male > female; $p < 0.025$).

Table 2. Peripheral blood MN-PCE temporal averages in benzene-exposed mice.

Strain	Sex	Route[a]	Exposure regimen	Control[b] Mean SEM	*n*	Exposed[b] Mean SEM	*n*
B6C3F$_1$	M	Gav	5 day	2.23 ± 0.200	9	24.04 ± 1.054*	9
B6C3F$_1$	M	Inh	5 day	2.42 ± 0.340	6	94.88 ± 3.211*	6
B6C3F$_1$	M	Inh	3 day	2.97 ± 0.229	6	99.90 ± 3.838*	6
C57B1/6	M	Inh	5 day	5.13 ± 0.233	6	104.32 ± 3.546*	6
C57B1/6	M	Inh	3 day	4.45 ± 0.281	6	100.26 ± 2.905*	5
DBA/2	M	Inh	5 day	2.05 ± 0.131	6	107.85 ± 5.529*	6
DBA/2	M	Inh	3 day	1.53 ± 0.150	6	121.23 ± 1.700*	6
DBA/2	F	Inh	5 day	1.55 ± 0.211	6	20.93 ± 3.173*	6
DBA/2	F	Inh	3 day	1.90 ± 0.188	6	24.40 ± 4.454*	6

[a]Abbreviations: gav, gavage; inh, inhalation.
[b]Mean number of MN-PCE per 1000 PCE ± SEM among *n* mice.
*Significantly different from corresponding control values at $\alpha = 0.05$.

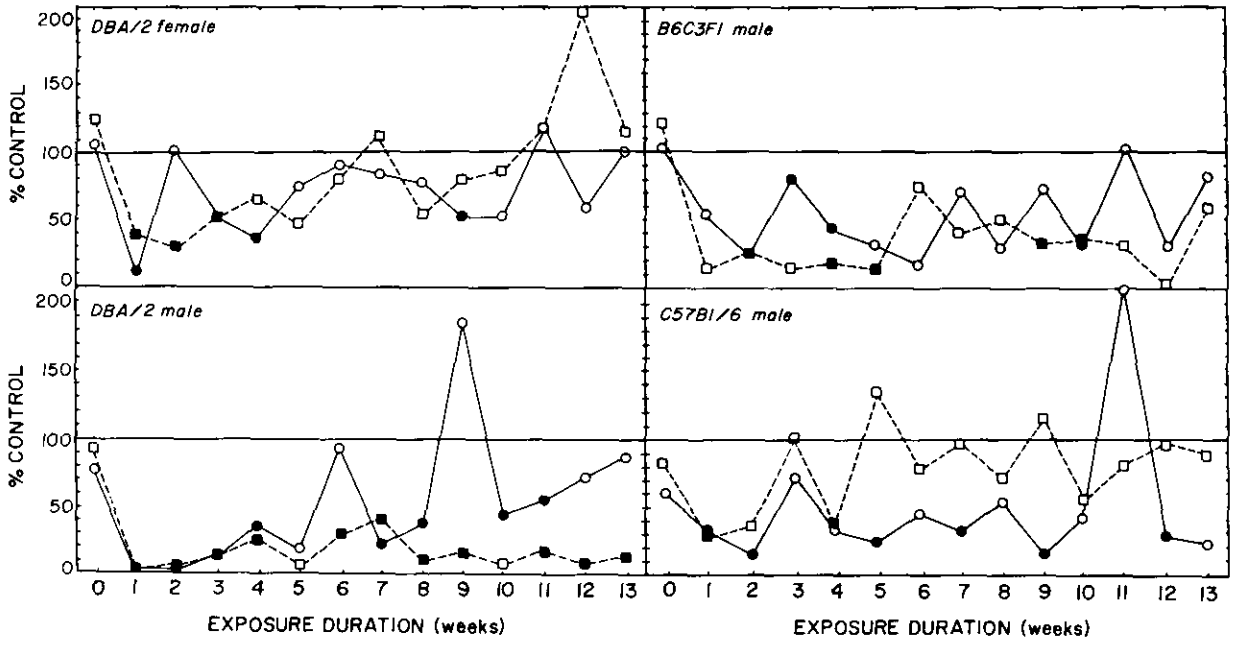

FIGURE 4. Group mean frequency of peripheral blood PCE across time in female DBA/2 mice, male DBA/2 mice, male B6C3F$_1$ mice, and male C57BL/6 mice exposed to 300 ppm benzene, presented as the percentage of the corresponding control group data. (□—□) Mice exposed 3 days per week; (○—○) mice exposed 5 days per week. Solid symbols indicate statistically significant differences between control and benzene-exposed mice as determined by Student's t-test at $\alpha = 0.05$.

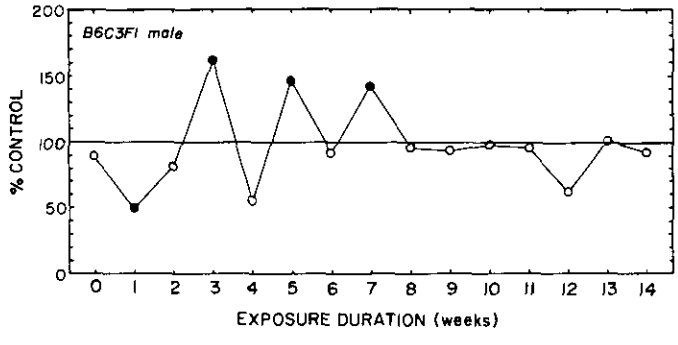

FIGURE 5. Group mean frequency of peripheral blood PCE across time in male B6C3F$_1$ mice administered 400 mg/kg benzene, presented as the percentage of the corresponding control group data. Solid symbols indicate statistically significant differences between control and benzene-exposed mice as determined by Student's t-test at $\alpha = 0.05$.

Bone Marrow Cellularity

Based on an analysis of cellularity in the tibia bone marrow of mice at the completion of the study period (Table 5), exposure to benzene significantly depressed the number of bone marrow cells in male ($p < 0.0001$) but only marginally so in female ($p = 0.0644$) mice. Among male mice, the extent of the depression depended on the route of exposure (inhalation > oral; $p = 0.0120$), but not on the inhalation exposure regimen ($p > 0.05$). Within the inhalation study, the depression in cellularity depended on the sex (male > female; $p = 0.0002$) and strain (DBA/2 < B6C3F$_1$ ≅ C57BL/6; $p = 0.0012$) of mice.

Discussion

The mouse peripheral blood MN assay is unique in that

Table 3. Peripheral blood percentage PCE temporal averages in benzene-exposed mice.

Strain	Sex	Route[a]	Exposure regimen	Control[b] Mean SEM	n	Exposed[b] Mean SEM	n
B6C3F$_1$	M	Gav	5 day	3.72 ± 0.159	9	3.53 ± 0.121	9
B6C3F$_1$	M	Inh	5 day	5.75 ± 0.598	6	2.76 ± 0.232*	6
B6C3F$_1$	M	Inh	3 day	7.58 ± 1.429	6	2.36 ± 0.515*	6
C57B1/6	M	Inh	5 day	5.13 ± 0.275	6	3.91 ± 0.455*	6
C57B1/6	M	Inh	3 day	5.41 ± 0.537	6	3.09 ± 0.533*	5
DBA/2	M	Inh	5 day	3.49 ± 0.152	6	1.48 ± 0.264*	6
DBA/2	M	Inh	3 day	3.56 ± 0.236	6	0.48 ± 0.072*	6
DBA/2	F	Inh	5 day	3.51 ± 0.129	6	2.40 ± 0.315*	6
DBA/2	F	Inh	3 day	3.83 ± 0.178	6	3.09 ± 0.520	6

[a]Abbreviations: gav, gavage; inh, inhalation.
[b]Mean percentage of PCE (based on analyzing 1000 erythrocytes) ± SEM among n animals.
*Significantly different from corresponding control values at $\alpha = 0.05$.

Table 4. Mean packed cell volume in benzene-exposed mice at the completion of the 13- to 14-week exposure period.

Strain	Sex	Route[a]	Exposure regimen	Control[b] Mean SEM	n	Exposed[b] Mean SEM	n	% Depression
B6C3F$_1$	M	Gav	5 day	48.3 ± 1.24	9	39.6 ± 0.90	9	18.0*
B6C3F$_1$	M	Inh	5 day	44.9 ± 2.94	4	30.7 ± 2.33	6	31.6*
B6C3F$_1$	M	Inh	3 day	38.9 ± 2.04	6	34.2 ± 0.77	5	12.1
C57B1/6	M	Inh	5 day	45.8 ± 2.19	6	36.5 ± 1.36	6	20.3*
C57B1/6	M	Inh	3 day	44.0 ± 1.18	6	21.9 ± 2.94	5	50.2*
DBA/2	M	Inh	5 day	48.0 ± 0.66	6	34.1 ± 3.00	4	29.0*
DBA/2	M	Inh	3 day	44.8 ± 0.96	6	29.8 ± 1.76	6	33.3*
DBA/2	F	Inh	5 day	47.8 ± 0.40	6	40.6 ± 1.52	6	15.2*
DBA/2	F	Inh	3 day	43.6 ± 1.01	6	37.8 ± 2.03	4	13.3

[a]Abbreviations: gav, gavage; inh, inhalation. PCV = packed cell volume
[b]Mean PCV (%) ± SEM among n animals.
*Significantly different at $\alpha = 0.05$.

Table 5. Mean tibia cellularity in benzene-exposed mice at the completion of the 13- to 14-week exposure period.

Strain	Sex	Route[a]	Exposure regimen	Control[b] Mean SEM	n	Exposed[b] Mean SEM	n	% Depression
B6C3F$_1$	M	Gav	5 day	7.29 ± 0.564	9	3.52 ± 0.405	9	51.7*
B6C3F$_1$	M	Inh	5 day	11.35 ± 1.207	5	4.41 ± 0.769	6	61.1*
B6C3F$_1$	M	Inh	3 day	14.97 ± 0.981	6	4.27 ± 0.183	5	71.5*
C57B1/6	M	Inh	5 day	14.91 ± 1.492	6	6.02 ± 0.417	6	59.6*
C57B1/6	M	Inh	3 day	14.68 ± 1.122	6	5.47 ± 0.346	5	62.7*
DBA/2	M	Inh	5 day	5.49 ± 0.540	6	0.85 ± 0.110	5	84.5*
DBA/2	M	Inh	3 day	6.71 ± 0.437	6	0.97 ± 0.122	6	85.5*
DBA/2	F	Inh	5 day	2.75 ± 0.377	6	1.94 ± 0.432	6	29.5
DBA/2	F	Inh	3 day	5.50 ± 0.935	5	2.31 ± 0.718	4	58.0*

[a]Abbreviations: gav, gavage; inh, inhalation.
[b]Mean number of nucleated cells per tibia ($\times 10^6$) ± SEM among n animals.
*Significantly different at $\alpha = 0.05$.

it enables, under multiple exposure conditions, a concurrent evaluation within the same animal of both acutely induced (i.e., within the last 1 to 2 days) and chronically accumulated (i.e., over 35 days) bone marrow damage (*16,17,30*). Thus, this assay is ideally suited for evaluating exposure duration-dependent alterations in bone marrow sensitivity to genotoxic agents. However, an interpretation of exposure duration-dependent alterations in bone marrow damage based on an analysis of MN-NCE only can be misleading because the frequency of micronucleated NCE is dependent not only on the frequency with which MN-PCE are induced but also on the rate of erythropoiesis, the transit time between enucleation of the erythrocyte precursor in the bone marrow and the identification of the cell as an NCE, and on the lifespan of both the normal and the micronucleated NCE under the study conditions (*31*).

Choy et al. (*12*) observed diminished levels of MN-NCE with increasing exposure duration in the peripheral blood of B6C3F$_1$ mice given benzene by gavage for 17 days, 54 days, and 103 weeks. In male CD-1 mice treated by gavage with benzene over an 8-week period, Barale et al. (*11*) observed a peak in MN-NCE frequency at about 5 weeks of exposure, followed by a decline in frequency over the remaining 3 weeks. Barale and his colleagues interpreted this lack of steady-state conditions to suggest either the selection of cells resistant to benzene, an increased detoxificaton of benzene, and/or a decreased ability of the animals to metabolize benzene. This interpretation is not unreasonable considering that metabolic studies on benzene have demonstrated an increased rate of benzene clearance/metabolism in rodents under repeated exposure conditions (*22,32–36*).

Theoretically, with multiple exposures, MN-NCE frequencies should attain steady-state conditions after the duration of the exposure period has exceeded the average NCE lifetime (i.e., some 5 to 7 weeks after the first exposure to benzene). In the present study, apparent steady-state conditions for MN-NCE levels were attained after about 5 weeks in female DBA/2 mice exposed to benzene by both regimens, in male mice of all three strains exposed to benzene for 3 days per week, and in male B6C3F$_1$ mice administered benzene by gavage on 5 days per week. However, in male mice exposed to benzene by inhalation for 5 days per week, the frequency of MN-NCE never attained steady-state conditions (Fig. 1).

Differences among the three strains in the kinetics of MN-NCE accummulation are readily apparent. In male DBA/2 mice, the frequency of MN-NCE continued to increase throughout the study, suggesting increased genotoxic sensitivity with increasing exposure duration. In male B6C3F$_1$ and C57BL/6 mice, the frequency of MN-NCE peaked around week 6, declined over the next 4 weeks, and then increased again near the end of the 13 week exposure period. The pattern of the MN-NCE response in the male mice of these latter two strains is quite

similar to that observed by Barale et al (*11*) for male CD-1 mice given benzene by gavage. The notable difference is that the duration of the study presented here was longer, permitting the detection of a second increase in the frequency of MN-NCE. The lack of steady-state conditions for MN-NCE frequencies suggests a duration-dependent alteration in the ability of benzene to induce genotoxic bone marrow damage in mice.

By evaluating the frequncy of MN-PCE throughout the course of the exposure period, this question of whether the ability of benzene to induce genotoxic damage (as defined by MN formation) in bone marrow is modulated by exposure duration can be examined critically. The lack of a time-dependent change in MN-PCE levels in the peripheral blood of benzene-exposed mice over the 13- to 14-week exposure period, regardless of the route of exposure or the exposure regimen, indicates that modulation of sensitivity and/or of metabolism did not alter the ability of benzene or its metabolites to induce bone marrow genotoxic damage. However, the age range over which this evaluation was conducted (i.e., 2–6 months) is relatively short, and, in more extensive studies (2–12 months of age), the age of the animal has been found to have a profound effect on the level of genotoxic and cytotoxic damage (as measured by the induction of sister chromatid exchanges and the inhibition of cellular proliferation kinetics) induced by a single exposure to benzene (*23*). Thus, more extensive studies may reveal an age-dependent modulation in benzene-induced bone marrow damage.

While the induction of MN-PCE was independent of exposure regimen, the exposure duration-dependent accumulation of MN-NCE was greater in mice exposed to benzene for 5 days per week than in mice exposed for 3 days per week. The difference in MN-NCE levels between regiments within a single sex/strain can be readily explained by the differences in the number of exposure days per week involved and by the generally greater suppression of erythropoiesis in mice exposed to benzene 3 days per week. That exposure to benzene suppresses the rate of erythropoiesis is not surprising in view of the large number of studies demonstrating the toxic effects of benzene on the hematopoietic system (*2,8,10,12*). The greater persistence of PCE suppression in male mice exposed to benzene for only three days per week was particularly striking. These data support the benzene metabolite studies of Irons (*18*) and provide evidence that the extent of benzene-induced toxicity is not simply related to the number of exposure days per week. The results of the analyses of PCV and bone marrow cellularity data collected at the completion of the exposure period reveal, with the single exception of bone marrow cellularity data for female DBA/2 mice, a significant depression in both end points among all groups of mice. However, while the cellularity data are always slightly more depressed in the mice exposed to benzene 3 days per week, the difference is not significant within mice of any sex or strain. The PCV data indicate a regimen-dependent difference in the magnitude of the depression induced by exposure to gaseous benzene, but only in DBA/2 mice. It is note clear why these various measures of bone marrow toxicity are not all in agreement. However, it must be remembered that the PCV and cellularity data were obtained at the completion of the exposure period and at a time when the percentage of PCE in the peripheral blood of benzene-exposed mice was not significantly different from control values.

Male DBA/2 mice exposed to benzene routinely demonstrated more genotoxic (MN induction) and cytotoxic (% PCE, PCV, cellularity) damage than did female DBA/2 mice. The increased level of damage in male mice is not unexpected, as there are numerous reports indicating that male mice are more sensitive to benzene than are female mice (*10*). The frequency of micronucleated erythrocytes in the peripheral blood of benzene-exposed mice also depended on the strain of mice. However, while male DBA/2 mice exhibited the highest frequency of MN-PCE, this strain exhibited the lowest frequency of MN-NCE. Considering the results of previous studies (*22,27*), the presence of a greater frequency of MN-PCE in male DBA/2 mice when compared to male C57BL/6 mice is not surprising. However, the fact that the differences in the MN-PCE temporal averages among the two strains was only approximately 10% is noteworthy. Harper and Legator (*28*), in a bone marrow MN study, reported that a single dose of benzene administered by gavage induced about a 1.5-fold greater increase in MN-PCE in male DBA/2 mice than in male C57BL/6 mice, a difference that increased following a course of multiple treatments. The different route of exposure used in these two studies probably accounts for the lack of agreement in these data. The strain-dependent difference in MN-NCE levels cannot be accounted for by the differences in MN-PCE frequencies among strains. Rather, the differences in the kinetics of MN-NCE accumulation observed for the three strains must result from the strain-specific effects of benzene on the rate of erythropoiesis. The strain with the greatest suppression also exhibited the slowest increase in MN-NCE levels. Males of all three strains exposed to gaseous benzene 5 days per week exhibited a similar depression in PCV values. Among mice exposed 3 days per week, DBA/2 males exhibited the greatest absolute decrease in PCV. However, this same strain exhibited the least absolute decrease in bone marrow cellularity among animals exposed for either 3 or 5 days per week.

Benzene, when administered by inhalation (300 ppm; 5 days per week, 6 hr per day), induced in male $B6C3F_1$ mice a much greater level of genotoxic (MN levels) and cytotoxic (% PCE, PCV, and bone marrow cellularity) damage than when given by gavage (400 mg/kg; 5 days per week). In this and in previous gavage studies (*11,12*), the frequency of MN-NCE in the peripheral blood of males treated with 500 mg/kg of benzene administered 5 days per week for some 8 to 17 weeks of treatment attained a level of 10 to 20 micronucleated cells per 1000 NCE. In contrast, the MN-NCE levels in $B6C3F_1$ mice exposed 5 days per week for 13 weeks to 300 ppm benzene attained a level of some 50 to 60 MN-NCE per 1000 NCE. The difference in the frequency of MN-PCE in mice exposed to benzene by the two different routes is

even more profound. Similarly, the depression in PCV and in bone marrow cellularity was much more severe in mice exposed to benzene by inhalation than in mice given benzene by gavage. This considerable difference in the level of genotoxic damage induced in the bone marrow of benzene-exposed mice depending on the route of exposure agrees with the pharmacokinetic studies of Henderson and co-workers (*37,38*). These investigators reported that much higher levels of the various metabolites of benzene can be achieved in the bone marrow when mice are exposed to benzene via the lungs than when it is administered by gavage.

The complex response of the mouse hematopoietic system to benzene depends on the sex and strain of the mouse, the route of exposure and, for some end points, on the duration and regimen of treatment. The results of these studies indicate that an evaluation of exposure duration-dependent changes in bone marrow sensitivity based simply on an evaluation of peripheral blood MN-NCE frequencies are inadequate. The observation that the depression in the rate of erythropoiesis (as measured by the percentage of PCE in peripheral blood) depended on the inhalation regimen needs to be confirmed and expanded. Such data clearly have implications for the extrapolation of animal data to human exposure situations. It is not obvious why suppression of erythropoiesis persisted for a longer duration in male mice exposed to 300 ppm benzene for fewer days per week. However, since proliferating cells appear to be more susceptible to the cytotoxic actions of benzene than are the normally quiescent stem cells and since benzene appears to inhibit stem cell proliferation, the 3-day exposure regimen probably provides a greater opportunity for toxicity (*8,18*). The significant dependence of the level of genotoxic and cytotoxic damage on the route of exposure suggests that the extrapolation of animal data from gavage studies to human occupational exposure situations may also be inadequate. The concentration of benzene (300 ppm) used in this study greatly exceed the current and recent occupational exposure limits. Furthermore, although 300 ppm benzene was believed to be within the linear portion of the dose range for the absorption of inhaled benzene in mice (*24*), more recent data (*37,38*) suggest that this dose lies above the linear portion of the dose-response curve for inhaled benzene metabolism. Thus, there is a need for additional studies on regimen- and route of exposure-dependent responses at lower concentrations of benzene.

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